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Novel HSP90 inhibitors effectively target thyroid cancer stem cell function preventing migration and invasion

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Abstract

Background—Thyroid cancer stem cells (CSCs) with ALDH and CD44 markers contribute to tumor growth and aggressiveness. We hypothesize that novel HSP90 inhibitors(KU711, WGA-TA) and 17-AAG can effectively target thyroid CSC function in vitro and prevent migration and invasion.

Methods—Validated papillary(TPC1), follicular(FTC238, WRO) and anaplastic(ACT1) human thyroid cancer cell lines were treated with three HSP90 inhibitors. CSCs were quantified for ALDH by flow cytometry(FC), CD44 expression by Western blot(WB), and thyrosphere formation assay. Cellular pathway proteins were analyzed by WB and migration/invasion by Boyden-chambers.

Results—WGA-TA and 17-AAG induced HSP70 compensation (not observed with KU711) on WB in all cell lines (>1,000 fold vs. controls). Only WGA-TA degraded HSP90-Cdc37 complexing by 60-70% vs. controls. Expression of HSP90 clients β -catenin, BRAF, Akt, and phospho-Akt were significantly inhibited by WGA-TA treatment(50-80%, 50-90%, >80%, and >90%) compared to controls, KU711, and 17-AAG treatment. KU711 and WGA-TA significantly reduced CD44 expression in all cell lines (25-60% vs controls/17-AAG), reduced ALDEFLOR activity by 69-98% ($p<0.005$) and sphere formation by 64-99% ($p<0.05$). Finally, cell migration was reduced by 31-98%, 100%, and 30-38%; and invasion by 75-100%, 100%, and 47% by KU711, WGA-TA, and 17-AAG treatment ($p<0.05$), respectively.

Conclusion—KU711 and WGA-TA are novel HSP90 inhibitors targeting CSC function and inhibiting cell migration/invasion in differentiated and anaplastic thyroid cancers, warranting further translational evaluation *in vivo*.

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Introduction

Thyroid cancer (TC) is the 9th most common malignancy overall and the most common endocrine malignancy¹. Although early stage differentiated TC (DTC) has an excellent prognosis, about 25% of patients develop recurrent disease, a third of which occur distantly or are RAI non-avid^{2,3}. Poorly differentiated and anaplastic thyroid carcinomas (ATC) have a worse prognosis due to their highly aggressive nature and limited therapeutic options. Unfortunately, for both advanced DTC and ATC, chemotherapy has not demonstrated long-term responses in a majority of patients, providing an opportunity for the development of novel therapeutics with improved outcomes⁴.

The cancer stem cell (CSC) hypothesis postulates that a small number of cells within a tumor have the ability of self-renewal, and may be responsible for tumor proliferation, invasion, metastatic potential, resistance to drug therapy, or the development of recurrent disease in TC^{5,6}. In TC, CSCs are identified primarily through aldehyde dehydrogenase (ALDH) activity and the surface marker CD44^{5,6}. The mitogen activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K)/Akt pathways have been shown to play integral roles in thyroid CSC characteristics^{6,7} in addition to the importance of their aberrant signaling in the pathogenesis of TC. The V600E BRAF mutation within the MAPK pathway is present in up to 40% of DTCs and causes constitutive activation of MAPK signaling, conveying a poorer prognosis, increased recurrence and decreased survival⁸. Additionally, PI3K/Akt activation (present in 24-55% of DTCs) leads to activation of mechanistic target of rapamycin (mTOR) to affect cell growth, proliferation and survival⁹.

Molecular chaperones such as heat shock protein 90 (HSP90) have been studied clinically as a potent anticancer therapy given their ability to control the activation of a number of “client” proteins involved in the pathways critical for cell growth, invasiveness and survival¹⁰. BRAF (MAPK pathway) and Akt (PI3K/Akt pathway) are two such HSP90 clients with key roles in TC growth and invasion,¹⁰ and HSP90 inhibitors such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) demonstrate down-regulation of these pathway proteins clinically¹¹. 17-AAG is a competitive inhibitor of the N-domain ATP-binding site on HSP90 leading to ubiquitination and proteosomal degradation of its client proteins. Although initially promising with clinical responses in early phase trials, it failed to progress past Phase II, in part due to poor solubility, dose-limiting toxicity and difficulty with formulations¹². Although subsequent 17-AAG derivatives improve upon formulation and toxicity limitations, all N-terminal HSP90 inhibitors share the same common pitfall. They induce HSP70 and HSP90 expression (pro-survival) at the same concentrations required for client protein degradation, thus creating a vicious cycle in which patients require higher and higher doses with increased frequency to overcome this HSP induction that ultimately pushes the patient toward the maximum tolerated dose and toxicity. As a consequence of these detriments, alternative methods to inhibit HSP90 are needed that do not induce this pro-survival, heat shock response, such as inhibiting the C-terminus of HSP90¹³⁻¹⁶.

In the present study, we evaluate two novel HSP90 inhibitors in TC. Compound KU711 is a novobiocin derivative and member of the coumermycin antibiotic family that binds to the

HSP90 C-terminal ATP-binding site, thereby disrupting complexing of co-chaperones and inhibiting allosteric conformational changes¹³⁻¹⁶. WGA-TA is a biosynthetic withanolide derived from the *Physalis longiflora* plant. Withanolides are a group of bioactive 28-carbon steroidal-lactones derived from the Solanacea plant family. While used for centuries in Aruveydic medicine, the isolated bioactive compounds, most notably withaferin A, demonstrate significant anti-angiogenic,¹⁷ anti-neoplastic effects through the formation of reactive oxygen species,^{18,19} inhibition of Akt and HSP90 clients to induce apoptosis,²⁰ and inhibition of epithelial-to-mesenchymal transition (EMT)²¹. Using structure activity relationships, our group created a 4,19,27-triacetate derivative of withalongolide A (WGA-TA) with higher potency and selectivity against cancer cells²². It has been proposed that withanolides inhibit HSP90 chaperone function through disruption of the HSP90-Cdc37 chaperone complex, which leads to degradation of client kinases that include regulatory kinases in the MAPK and PI3K/Akt pathways²³ important in TC tumorigenesis and stem cell function. We hypothesize that our novel C-terminal HSP90 inhibitor (KU711) and HSP90-Cdc37 complex inhibitor (WGA-TA) down regulate the expression of HSP90 client proteins in TCs, including their stem cells, leading to effective inhibition of thyroid CSC function as noted by decreased proliferation, thyrosphere formation and invasion *in vitro*.

Materials and Methods

Cell Culture/Reagents

Validated human TC cell lines TPC1 (papillary), FTC238 and WRO (follicular) and ACT1 (anaplastic) were graciously obtained from Dr. Sonia Sugg (University of Iowa, Iowa City, IA). TPC1 has a RET/PTC rearrangement and Ras mutation, WRO is a V600E BRAF mutant, and FTC238 is from a lung metastasis with complex chromosomal changes and p53 mutation. Cells were maintained in 2-D culture in high glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Lewis, MO), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY) according to our labs previously published methods²⁰. Drug compounds utilized for these experiments included the novel C-terminus HSP90 inhibitor KU711 obtained from Dr. Brian S. J. Blagg (University of Kansas, Lawrence, KS), withalongolide A 4,19,27-triacetate (WGA-TA) which was isolated and purified by Dr. Barbara Timmermann (University of Kansas, Lawrence, KS), and the N-terminus HSP90 inhibitor 17-N-allylamino-17-demethoxygeldanamycin (17-AAG, Sigma-Aldrich, St. Lewis, MO).

Western Blot Analysis (WB)

When cells reached 60-80% confluence, they were treated for 24h at 20 or 40 µM KU711 (1 and 2x IC₅₀ for TC), 1-5 µM WGA-TA (1-5x IC₅₀ for TC), or 2 µM 17-AAG (2-3x IC₅₀ for TC). Following treatment, cells were collected and lysates prepared for WB by re-suspending cells in lysis buffer with protein concentrations determined using the BSA protein assay (Thermo Fischer Scientific, Waltham, MA). Membrane loading and blotting was performed as per previously published methods from our lab²⁰. Akt, P-Akt, CD44, Cdc37, BRAF, E-cadherin, ERK1/2, and phospho-ERK1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA), HSF1, HSP90, and HSP70 from Enzo Life

Sciences (Farminale, NY), β -Actin from EMD Millipore (Billerica, MA), β -catenin from Thermo Fisher Scientific (Waltham, MA), and donkey anti-rabbit IgG HRP (1:5000) and goat anti-mouse IgG HRP (1:5000) secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were then treated with SuperSignal West PICO or FEMTO (Thermo Fischer Scientific, Waltham, MA) for 5 min and visualized by enhanced chemiluminescence and captured on autoradiography film (Molecular Technologies, St. Lewis, MO) on a Konica Minolta SRX 101A developer (Ramsey, NJ). Actin levels were assessed to ensure equal loading and transfer of proteins. All studies were repeated for accuracy. ImageJ software (National Institutes of Health, Bethesda, MD) was used to obtain relative density of protein bands after WB analysis.

ALDEFLOUR Assay

FTC238 cells were treated and collected in the manner outlined for WB analysis, then evaluated for ALDH activity using ALDEFLOUR assay kit as per the manufacturer's instructions (STEMCELL technologies, Vancouver, BC, Canada). Flow cytometric analysis was conducted on a CyAn ADP analyzer (Beckman Coulter, Brea, CA). DAPI staining was used to exclude dead cells from analysis by gating, and used to assess treatment related cell viability.

Thyrosphere Formation

FTC238 and ACT1 cells were plated at 100 cells/well in a 96-well ultralow attachment plate (Corning, Corning, NY) in DMEM/F12 with 1x B27 (Life Technologies, Grand Island, NY), 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor (both BD biosciences, Bedford, MA) with varying concentrations of KU711, WGA-TA and 17-AAG. After 14 days of culture, spheres (>25 cells) per well were counted using light microscopy.

Migration and Invasion Assay

At least 2 h prior to use, 8-micron polycarbonate Boyden chambers (Corning, Corning, NY) were coated with 100 μ L of 300 μ g/mL of reduced growth factor Matrigel (Corning, Corning, NY) and incubated for 2h at 37° C. Each cell line was resuspended in serum free DMEM with penicillin/streptomycin and 20 or 40 μ M KU711, 1 or 2.5 μ M WGA-TA, or 2 μ M 17-AAG and equal numbers of cells were plated onto either standard or Matrigel coated upper wells with 10% FBS/DMEM as lower well chemoattractant. Chambers were incubated for 20h at 37° C, migrated cells were fixed in 2% paraformaldehyde, and stained with 1% crystal violet in 20% methanol for 20 min. Next, the membranes were washed in Millipore water, and the remaining upper-well cells were removed with a cotton-tipped swab. Migration using standard chambers and invasion with Matrigel coated chambers were quantified as number of cells per high-powered field.

Statistical Analysis

Students two-tailed, unpaired t-test was used to identify significance (set as 95%, $p < 0.05$) between treatment groups in FC, thyrosphere assays and Boyden chamber assays. Migration and invasion data was proportionally adjusted for treatment related cell death using

corresponding viability data from DAPI exclusion by FC. Data are presented as mean values with error bars denoting standard deviation. Experiments were replicated to ensure accuracy.

Results

Effect of HSP90 inhibitors on HSP70 compensatory induction and Cdc37 expression

To evaluate heat shock response following treatment with HSP90 inhibitors, heat shock proteins HSP90, HSP70 and the co-chaperone Cdc37 were evaluated by WB following 24h treatment with KU711, WGA-TA and 17-AAG in the TPC1, FTC238, WRO and ACT1 cell lines (Figure 1). While HSP90 remained relatively stable with either KU711 or WGA-TA treatment, 2 μ M 17-AAG treatment increased HSP90 expression by 50% across all cell lines. WGA-TA and especially 17-AAG treatment lead to a significant compensatory pro-survival response with up-regulation of HSP70 levels across all cell lines, not seen with KU711 treatment. Additionally, in FTC238 and ACT1 cells, we demonstrate no change in the reporter HSF1 expression with KU711 or 17-AAG treatment, whereas there was dose dependant reduction in HSF1 with WGA-TA, with almost complete inhibition at 2.5 μ M (>95% reduction). HSF1 expression was not constitutively expressed in TPC1 and WRO cell lines, and we see a significant increase in HSF1 expression with KU711 treatment, which was not seen with other HSP90 inhibitors. Lastly, with WGA-TA treatment, there was a significant reduction in HSP90-Cdc37 complexing across all cell lines (41-96% reduction in Cdc37:HSP90 ratio), not observed with KU711 or 17-AAG treatment.

Effect of HSP90 inhibitors on oncogenic and cancer stem cell maintenance proteins

We next evaluated the effect of HSP90 inhibition on TC stem cell maintenance pathways including MAPK and PI3K/Akt (Figure 2). Following treatment with KU711, WGA-TA and 17-AAG, representative MAPK proteins BRAF, ERK and phospho-ERK were evaluated by WB. Treatment with 40 μ M KU711 showed a 56% and 66% reduction in BRAF expression in TPC1 and ACT1 cells, respectively. In a concentration dependent manner, WGA-TA reduced BRAF expression by 21-56% in TPC1 and FTC238 cells with complete inhibition in WRO cells. This was in stark contrast to the 42-116% increase in BRAF expression across all cell lines noted following 17-AAG treatment. Looking at the nuclear protein ERK, downstream of BRAF, phosphorylation of ERK was increased by WGA-TA in all cell lines and in ACT1 cells by KU-711 without any effects noted with 17-AAG treatment. Next, we evaluated the PI3K/Akt pathway as well as β -catenin expression. In the non-canonical pathway, Akt activates β -catenin, which is implicated in CSC self-renewal, proliferation and development²⁴. Functional phospho-Akt levels were inhibited by 33-99%, with WGA-TA being the most potent. WGA-TA treatment lead to 30-85% reduction in β -catenin expression levels compared to untreated levels, or cells treated with KU711 or 17-AAG ($p<0.01$).

KU711 and WGA-TA treatment reduce CSCs in a dose dependent manner

Because KU711 and WGA-TA showed significant reductions in BRAF expression and WGA-TA showed inhibition of the MAPK, PI3K/Akt and β -catenin pathways, we next evaluated these TC cells for CSC markers using CD44 expression, ALDH activity (Figure 3a-c), and thyrosphere formation (Figure 4). After 24 h treatment with 20-40 μ M KU711, 1-2.5 μ M WGA-TA and 2 μ M 17-AAG, we evaluated CD44 expression by WB and ALDH

activity by ALDEFLOUR assay. KU711 and WGA-TA treatment resulted in a dose dependent reduction in CD44 expression in all three DTC cell lines, with maximal reductions of 30-51% with 2.5 μ M WGA-TA treatment, whereas a more variable response with 17-AAG. Next, ALDEFLOUR assay was used to evaluate ALDH activity. In TPC1/WRO and ACT1 cell lines, baseline activity was less than 0.5%, making an accurate evaluation of treatment effects difficult (data not shown). However, FTC238 showed 11.4% baseline ALDH positivity (Figure 3b and c), and both KU711 and WGA-TA show a significant concentration dependant reduction in ALDH activity (69-89% for KU711 vs. controls [$p<0.005$] and 83-98% for WGA-TA [$p<0.005$ vs. controls and $p<0.05$ vs KU711 and 17AAG]). 17-AAG did not significantly inhibit ALDH activity vs. controls ($p=0.06$). Lastly, thyrosphere formation assays with FTC238 and ACT1 cell lines demonstrated a dramatic decrease in sphere formation following 14 days treatment with KU711, WGA-TA and 17-AAG (Figure 4). At 5 μ M KU711 for ACT1 and 10 μ M for FTC238 sphere formation was significantly inhibited by 63.6% ($p<0.05$) and 99.6% ($p<0.005$), respectively. At only 62.5 nM WGA-TA treatment, there are 83.1% and 84.8% reductions in sphere formation for FTC238 and ACT1, respectively ($p<0.01$ vs control), and at 0.25 μ M 17-AAG sphere formation was reduced by 94.4% and 75.8% for FTC238 and ACT1, respectively ($p<0.05$ vs control).

Inhibition of key CSC maintenance pathways with decreases in migration and invasion

To examine whether reduced levels of CSCs leads to decreases in invasive potential, we analyzed migration, invasion, and the expression of the epithelial differentiation marker E-cadherin, which is indicative of reduced EMT. Using Boyden chambers, migration and invasion was determined after 20 h treatment KU711, WGA-TA and 17-AAG. Because it can be difficult to differentiate cellular death effects due to treatment from decreases in migration and invasion, subsequent migration and invasion data was adjusted proportionally to DAPI exclusion viability data obtained from FC (data not shown). Across ACT1, FTC238 and TPC1, there was a significant dose-dependent decrease in both migration and invasion with KU711 and WGA-TA treatment (Figure 5a-b). WGA-TA had the most potent effect, inhibiting both invasion and migration by 90-100% in all cell lines ($p<0.005$ vs. controls). KU711 inhibited migration by 31-98% across all cell lines ($p<0.05$ – $p<0.005$), and invasion by 75-100% ($p<0.005$) compared to controls. 17-AAG treatment also reduced migration (33-47% vs controls, $p<0.05$), with only modest but significant inhibition of invasion in the TPC1 cells (38% vs control, $p<0.05$). Comparing the effect of different HSP90 inhibitors, WGA-TA was the most potent with significant inhibition in both invasion and migration compared to 17-AAG ($p<0.05$) and to 20 μ M KU711 ($p<0.02$), while 40 μ M KU711 significantly inhibited invasion and migration compared to 17-AAG ($p<0.05$). Subsequently, we evaluated E-cadherin expression as a marker of epithelial differentiation and decreased EMT. In ACT1 cells, E-cadherin expression was increased by 440% with WGA-TA and 180% with KU711 treatment compared to controls, whereas 17-AAG had no change in E-cadherin levels ($p<0.01$) (Figure 5c).

Discussion

Because of the stark differences in survival between early stage and advanced TC, the search continues for more effective clinical treatments for these patients. While multi-receptor tyrosine kinase inhibitors (TKIs), such as sorafenib and recently lenvatinib, are FDA approved for the treatment of advanced DTC, neither has demonstrated a significant improvement in overall survival. Because 90 kDa heat shock proteins (HSP90) act as molecular chaperones responsible for folding many proteins directly associated with malignant progression, they represent one of the most promising biological targets identified for the treatment of cancer. Inhibition of the HSP90 protein folding machinery would result in a combinatorial attack on numerous oncogenic pathways. Furthermore, studies have revealed that HSP90 inhibitors accumulate in tumor cells much more efficiently than in normal tissue, leading to a differential selectivity of ~200 fold¹⁰. Although no HSP90 inhibitors have progressed past clinical trials, they demonstrate significantly down-regulation of MAPK and PI3K/Akt pathway proteins,¹¹ and pave the way to search for more efficacious and less toxic HSP90 inhibitors.

Our group has focused on two novel inhibitors of the HSP90 heterochaperone complex. KU711, a C-terminal HSP90 inhibitor, and WGA-TA, a biosynthetic withanolide that blocks the binding of the Cdc37 co-chaperone (that activates client kinase proteins) to the HSP90 heterochaperone complex. In looking first at the heat shock response to treatment, we found HSP90 and HSP70 levels were induced by 17-AAG as previously reported¹⁵ that the C-terminal HSP90 inhibitor KU711 did not induce this compensatory pro-survival HSP70 up-regulation, which may be related to its ability to inhibit HSP70 complexing to HSP90^{13,14}. While WGA-TA treatment did induce HSP70 expression, it had no significant effect on HSP90 levels and completely inhibited expression of HSF1, indicating a somewhat different effect on heat shock response and modulation of the heterochaperone complex compared to the other two inhibitors. Under normal stress conditions, HSF1 leaves the HSP heterocomplex to trimerize, localize to the nucleus, and enact downstream pro-survival pathways through DNA transcriptional binding. WGA-TA inhibition of these downstream pro-survival pathways may play a role in its robust cancer cell death. Although KU711 appeared to induce HSF1 expression in WRO and TPC1 cells, this did not induce HSP70, a known HSF1 target gene and demonstrates the multifactorial nature of our HSP90 inhibitors. Together, KU711 and WGA-TA each decrease normal heat shock pro-survival responses within separate pathways not observed with 17-AAG, suggesting these inhibitors may be able overcome some of the challenges observed with N-terminal inhibitors. Finally, WGA-TA decreased Cdc37 levels consistent with its mechanism of blocking this co-chaperone interaction with HSP90, and this mechanism could explain how WGA-TA selectively inhibits multiple key regulatory kinases in cancer cells including Akt and BRAF in TC cells. Unlike 17AAG, which induced BRAF expression and had small effects on Akt, KU711 inhibited both BRAF and phospho-Akt expression, while WGA-TA potently inhibited BRAF, Akt, phospho-Akt, and β -catenin. While downstream of BRAF, WGA-TA treatment lead to hyper-phosphorylation of ERK, which has been previously described in other cancer models to be a compensatory pro-survival response to apoptosis²⁰. ERK modulation identifies a potential target for combination use with WGA-TA to enhance its efficacy.

Together, these effects on the MAPK and PI3K/Akt pathways are important in inhibiting TC growth as well as thyroid CSC functionality.

Given that KU711 and WGA-TA inhibited pathways that are also important to TC stem cell function, we evaluated the effect of treatment on the thyroid CSC population via CD44 expression by WB, ALDH activity by ALDEFLOUR assay, and thyrosphere formation. Treatment with both KU711 and WGA-TA reduced CD44 expression, ALDH activity and thyrosphere formation in a concentration dependent manner when compared to controls, with WGA-TA having the most potent CSC targeting effects. While the treatment effects on ALDH activity could not be assessed in our additional cell lines due to low baseline ALDH activity, we did demonstrate a significant reduction in thyrosphere formation, a hallmark of CSCs, in multiple cell lines with all three HSP90 inhibitors at low therapeutic concentrations. Since thyrospheres are felt to have enriched CSC populations and have been shown to form tumor xenografts from a smaller number of cells compared to bulk cell lines in monolayer culture,²⁵ the reductions in CD44 expression, ALDH activity and thyrosphere formation indicate that the surviving TC cell populations are significantly depleted of CSCs, and that CSCs are susceptible to HSP90 inhibition. Next, due to the role of CSCs on tumor invasion, migration, and EMT, we evaluated our inhibitors' effect on these processes. Both KU711 and WGA-TA demonstrated a significant reduction in both migration and invasion in a concentration dependent manner compared to control and 17-AAG, again with the most potent effect observed with WGA-TA. Furthermore, E-cadherin expression was increased with KU711 and WGA-TA treatment compared to both control and 17-AAG treatment, indicative of an epithelial phenotype and inhibition of EMT by these two inhibitors.

These results demonstrate that novel inhibitors of the HSP90 heterochaperone complex such as KU711 and WGA-TA act differently than 17-AAG, which avoids some of its limitations including induction of HSP70 and MAPK. Because 17-AAG is a pan-HSP90 inhibitor that completely blocks all HSP90 clients, it has the most robust induction of pro-survival HSPs, which subsequently diminishes 17-AAG's effect on downstream outcomes like invasion and migration. Conversely, KU711 and WGA-TA are partial inhibitors that affect fewer, though still essential, HSP90 clients, which results in a blunted pro-survival HSP response. In this manner, our novel inhibitors have a greater functional affect compared to 17-AAG. Additionally, these new compounds can selectively inhibit thyroid CSC function and even reduce their population *in vitro* with decreases in cell growth, invasion, migration, and even EMT. As such, these promising agents warrant additional evaluation to further elucidate their mechanism related to thyroid tumor biology, as well as how their effects on thyroid CSCs *in vitro* translate into *in vivo* effects on tumor growth, aggressiveness and metastatic potential. Completion of these key preclinical studies may lead to important new therapeutic strategies for advanced TCs to improve treatment durability and combat drug-resistance mechanisms in the future.

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Figure 1.

Western blot of heat shock proteins (HSP) in thyroid cancer cell lines FTC238, WRO, ACT1 and TPC1. Cells were treated for 24 h with 20-40 μM KU711, 1-5 μM withalongolide A 4,19,27-triacetate (WGA-TA) or 2 μM 17-(allylamino)-17-demethoxygeldanamycin (17-AAG). Cell lysates were collected and Western blotted for HSP90, HSP70, heat shock factor 1 (HSF1) and Cdc37. There is 50% increase in HSP90 expression with 2 μM 17-AAG treatment across all cell lines, whereas they are relatively stable in KU711 and WGA-TA. In WGA-TA and especially 17-AAG treatment, HSP70 levels increase by >1250%, which is not seen with KU711 treatment. In FTC238 and ACT1 cells, HSF1 remained unchanged, whereas WGA-TA treatment reduced HSF1 expression in a dose-dependent manner, with almost complete inhibition at 2.5 μM concentration (>95% reduction). With 1-5 μM WGA-TA treatment, Cdc37-HSP90 complexing was reduced across all cell lines (41-96% reduction in Cdc37:HSP90 ratio), not observed with KU711 or 17-AAG treatment.

Figure 2.

Western blot of mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K)/Akt pathway proteins in thyroid cancer stem. Cells were treated in a similar manner as described in Figure 1. Treatment with 40μM KU711 showed a 56% and 66% reduction in BRAF expression in TPC1 and ACT1 cells, respectively, while WGA-TA reduced BRAF expression by 21-56% in TPC1 and FTC238 cells with complete inhibition in WRO cells. In contrast, 17-AAG treatment resulted in a 42-116% increase in BRAF expression across all cell lines. Phosphorylation of nuclear protein ERK was increased by WGA-TA in all cell lines, and in ACT1 cells by KU-711 without any effects noted with 17-AAG treatment.

Functional phospho-Akt levels were inhibited by 33-99% by all three inhibitors with WGA-TA being the most potent. WGA-TA treatment lead to 30-85% reduction in β -catenin expression levels compared to untreated levels, or cells treated with KU711 or 17-AAG ($p<0.01$).

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Figure 3.

Effects of KU711, WGA-TA and 17-AAG treatment on CD44 expression and aldehyde dehydrogenase (ALDH) activity. FTC238, WRO and TPC1 cells were treated with each HSP90 inhibitor in a similar manner as described in Figure 1. ALDH activity was assessed by ALDEFLOUR assay and flow cytometry. 3a. KU711 and WGA-TA treatment decreased CD44 expression levels in a dose dependent manner, with maximal reductions at 2.5 μM WGA-TA (30-51%) across all cell lines. 17-AAG treatment varied, with reduction of CD44 in TPC1 cells and an increase in FTC238 and WRO cells. 3b. Graph plot of ALDH positive cells by ALDEFLOUR assay. KU711 treatment reduced ALDH activity by 69-89% ($p < 0.005$ vs controls), whereas WGA-TA reduced activity by 83-98%, which was significant compared to controls ($p < 0.005$) and to KU711 ($p < 0.05$). 17-AAG did not have significant inhibition. 3c. Representative dot plots of ALDEFLOUR assay with inhibitory DEAB as negative control. Controls demonstrate 11.4% baseline ALDH activity, with KU711 treatment reducing activity to 3.56% and 1.06% with 20 and 40 μM, respectively. WGA-TA treatment at 1 and 2.5 μM reduced ALDH activity to 2.17% and 0.28%, respectively. Treatment with 17-AAG was not significant. * signifies $p < 0.05$, ** signifies $p < 0.005$.

Figure 4.

Thyrosphere formation in FTC238 and ACT1 cells. Cells were plated at 100 cells/well in sphere medium in ultra-low adhesion plates and treated with various concentrations of KU711, WGA-TA and 17-AAG for 14 days. 4a. Representative images of thyrospheres. 4b. Bar graph representation of average thyrosphere numbers per well in each treatment group. There is a significant concentration dependent decrease in sphere formation with KU711 treatment, with 99.6% reduction at 10 µM for FTC238 ($p < 0.005$) and 63.6% reduction at 5 µM for ACT1 ($p < 0.05$). At only 62.5 nM WGA-TA treatment, there are 83.1% and 84.8% reductions in sphere formation for FTC238 and ACT1, respectively ($p < 0.01$). For 17-AAG, at 0.25 µM concentration, there are 94.4% and 75.8% reductions in sphere formation for FTC238 and ACT1, respectively ($p < 0.05$). * signifies $p < 0.05$, ** signifies $p < 0.005$.

Figure 5.

Migration, Invasion and epithelial-mesenchymal transition (EMT) in ACT1, FTC238 and TPC1 cells. Cells were treated for 20 hours in a similar manner described in Figure 1. Migration measured on standard membranes while invasion was measured on membranes coated with Matrigel. Media with 10% fetal bovine serum was used as chemoattractant in the lower chamber. 4a. Representative images of migration and invasion for ACT1 cells. 4b. Graph plot of the number of cells counted per high powered field corrected for cell viability to partially account for treatment related cell death. WGA-TA treatment demonstrated >90% inhibition of both migration and invasion in all cell lines ($p<0.005$ vs controls). KU711 treatment in all cell lines resulted in 31-98% and 75-100% inhibition of migration and invasion, respectively ($p<0.05$ - $p<0.005$). Treatment with 17-AAG reduced migration by 33-47% in all three cell lines ($p<0.05$ vs controls), and only inhibited invasion in TPC1 (38% vs control, $p<0.05$). WGA-TA was the most potent HSP90 inhibitor, with significant inhibition of both migration and invasion compared to 20 μ KU711 and 17-AAG treatment ($p<0.05$), while 40 μ KU711 significantly inhibited invasion and migration compared to 17-AAG ($p<0.05$). 4c. E-cadherin expression in ACT1 cells. There is 180% increase in expression with 40 μ KU711 treatment, and 440% increase in expression following 5 μ WGA-TA treatment, whereas 17-AAG treatment had no effect ($p<0.01$). * signifies $p<0.05$, ** signifies $p<0.005$.